THE APPLICATION OF DIAGNOSTICS IN PLANT HEALTH VIROLOGY

by

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Declaration

This thesis is the result of my own work. Where work is the result of a collaboration, the individual contributions of the author are specifically listed within Appendix 2 and these are accompanied by signatures or supporting letters from co-authors.

It has not been previously submitted, in part or whole, to any university or institution for any degree, diploma, or other qualification.

In accordance with the University of Warwick guidelines for submission of a thesis for PhD by published works, the linking text (Introduction and Discussion/Conclusions) does not exceed 10,000 words.

Signed:

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Summary

Using conventional diagnostic methods to test for plant viruses requires knowledge of the pathogens likely to be associated with a host species. This knowledge can either be applied when using a targeted test method (e.g. ELISA or PCR) to identify required tests, or in bioassay, through knowing which viruses will transmit into which assay host. In the last decade High Throughput Sequencing (HTS) has revolutionised plant virology diagnostics, however, the knowledge and skills of the diagnostic virologist are needed to interpret the results of sequencing and to investigate the myriad of novel viruses reported using this technique. This thesis presents a body of published work and an accompanying linking document focussing on the development and application of these diagnostic technologies in a plant health/biosecurity setting. The thesis explores the use of diagnostic technologies in virus detection and discovery, but also in supporting research applications such as gathering the data necessary to support plant health risk assessment or carry out epidemiological studies on vector efficiency. The publications include a review of new virus records from the United Kingdom over a 35-year period, discussing the factors driving virus discovery such as changes in trade, research focus, and diagnostic technologies. Two case studies are presented which investigate diseases of unknown aetiology utilising contrasting approaches to infer the causal agent/s of disease, one utilising biological demonstration, the other experimental design and statistical analysis. Two publications discuss the evaluation and validation of diagnostic techniques. The final publication describes an investigation into the relative efficiency of transmission of potato virus Y and potato virus A by a range of aphid species. The accompanying linking document discusses each publication in the context of the current literature, as well as discussing alternative approaches to inferring causation where traditional biological approaches may not be possible.

List of Abbreviations and Acronyms

AHDB	Agriculture and Horticulture Development Board
AOD	Acute Oak Decline
CaTV-1	Carrot torrado virus-1
cDNA	complementary DNA
CIMMYT	International Maize and Wheat Improvement Center
CLVd	Columnea latent viroid
CMD	Carrot Motley Dwarf disease
CtRLV	Carrot red leaf virus
CYLV	Carrot yellow leaf virus
DIG-Probes	Digoxigenin-labelled probes
DNA	Deoxyribonucleic acid
EC	European Community
EFSA	European Food Standards Authority
ELISA	Enzyme Linked Immunosorbant Assay
OEPP/EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
HTS	High Throughput Sequencing
IITA	International Institute of Tropical Agriculture
IPPC	International Plant Protection Convention
ISO	International Organization for Standardization
ISPM	International Standards of Phytosanitary Management
LAMP	Loop-mediated isothermal Amplification

LNLCV	Lettuce necrotic leaf curl virus
LoD	Limit of Detection
MCMV	Maize chlorotic mottle virus
MLN	Maize Lethal Necrosis disease
MYMoV	Motherwort yellow mottle virus
NASH	Nucleic Acid Spot Hybridisation
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
PLRV	Potato leaf roll virus
PMS	Pansy Mottle Syndrome
PRA	Pest Risk Analysis
PSTVd	Potato spindle tuber viroid
PVA	Potato virus A
PVY	Potato virus Y
PYFV	Parsnip yellow fleck virus
REF	Relative Efficiency Factor
RNA	Ribonucleic Acid
RPKM	Reads Per Kilobase Million
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SASA	Science and Advice for Scottish Agriculture
SCMV	Sugarcane mosaic virus
TEM	Transmission Electron Microscope
UK	United Kingdom
VWDaV	Viola white distortion associated virus

1 LINKING DOCUMENT: INTRODUCTION AND DISCUSSION

1.1 Introduction

Historically, plant pests and diseases have been responsible for famine, mass migration, and societal change as a consequence of their impact upon food security. Between 1845 and 1849 the Irish potato famine, caused by late blight of potato *Phytophthora infestans,* killed or displaced around 25% of the population of Ireland (Fraser, 2003). The same pathogen caused failures in potato crops across Europe. Concurrent failures in cereal harvests caused by a combination of factors including fungal pathogens, also caused famine, mass-migration, and (arguably) civil unrest in mainland Europe (Zadoks, 2008). Similarly, in 1943 an estimated 2 million people died in Bengal, India, as a result of shortages in the rice harvest due to brown spot of rice *Bipolaris oryzae* (synonym *Helminthosporium oryzae*), compounded by failures in contingency response by the civil administration (Padmanabhan, 1973). Aside from these high profile historic case studies, the impact of plant pests (*sensu lato*), and more specifically plant viruses, as a limiting factor for food security is not fully appreciated in societies with abundant food supplies. Losses will be unevenly distributed and likely to have greater impact in areas of poor food security (Savary et al., 2019). Strange and Scott (2005) noted that just 14 crops constitute the majority of global food supply and estimated that diseases were responsible for approximately a 10% reduction in yield. Estimates for losses in specific crops related to viral infections are *circa* 7%, 3%, and 2.5% for potatoes, maize and wheat respectively (Oerke, 2006). Notable examples of current food security issues caused by viral pathogens include the double constraints to production of cassava (*Manihot esculenta*), namely cassava brown streak disease (Fig 1a) and cassava mosaic disease (Fig 1b) (Legg et al., 2015) and the rapidly emerging problem of maize lethal necrosis (Fig 1c) in East Africa (Wangai et al., 2012, Adams et al., 2013, Mahuku et al., 2015a).

Figure 1 (a) Internal necrosis in tubers of cassava (*Manihot esculenta*) as a result of infection with cassava brown streak virus. (b) chlorotic mosaic and leaf deformation of cassava as a result of infection with cassava mosaic virus. (c) Unaffected (tolerant) (left) and affected (right) maize (*Zea mays*) infected with the virus complex maize lethal necrosis disease. Images reproduced under creative commons (a) and (b) IITA Image library image (c) CIMMYT Image library

Plant health is the term given to the regulatory control of plant pests and diseases with the aim of preventing outbreaks through the implementation of biosecurity measures. Plant health systems are guided by a series of standards implemented under the International Plant Protection Convention, that aims to “secure coordinated, effective action to prevent and to control the introduction and spread of pests of plants and plant products”. These are known as the International Standards on Phytosanitary Measures (ISPMs). The decision on the regulatory status of a given plant pest and the basis of control actions are governed by plant health pest risk analysis (PRA), the international standards for conducting PRAs are laid out in the ISPM guidelines for pest risk analysis (ISPM, 1996). Each step of this process from initiation, through the key considerations for pest or pathogen entry, establishment, and spread, through to identification of mitigation measures for pest risk management are all underpinned by the need for accurate diagnostics. Generally, the application of these diagnostic methods can be separated into ‘detection’ and ‘diagnosis’. Detection is the application of a diagnostic method to indicate the presence of a pest or disease in a given area, consignment in trade, or individual plant, whereas diagnosis is the use of that method as part of a broader multiphasic process to identify the causal agent of a malady, or

confirmation of the taxonomic identity of a previously detected pathogen (Adams et al., 2009).

The appropriate interpretation of any test method relies on understanding the performance characteristic of each method for a given application through gathering specific supporting data, a process termed ‘validation’. These validation data inform the level of confidence in a positive or negative result. With the exception of ‘screening’ tests, diagnostic testing is generally performed as a multiphasic process, with a cascading array of tests from a suite of methods applied to a sample to determine the causal agent of a disease, or to detect and then identify the pathogen present. Each of these tests should be validated in line with their purpose in the diagnostic process (Roenhorst et al., 2018). This validation at the systems level is then supported by use of appropriate test controls and the expertise and knowledge accumulated within the diagnostic laboratory (Chabirand et al., 2016, Roenhorst et al., 2018). Screening tests may be integrated into a system as described above, however, they may also be used as stand-alone methods.

Plant viruses present a diagnostic challenge due to their obligate nature. These pathogens cannot be cultured outside of a suitable host, and therefore cannot be isolated in pure cultures on artificial media as with bacterial and fungal pathogens. Consequently, virologists have had to rely on methods for the direct detection of pathogens from host plants. These detection methods can be brigaded into two groups namely ‘targeted’ and ‘non-targeted’ methods (Adams et al., 2009, Fox & Mumford, 2017). Briefly, targeted methods are designed to detect specific target pathogens, and may be designed to detect specific strains, species or groups of species. Targeted methods include serological methods e.g. Enzyme Linked Immunosorbent Assay (ELISA), or nucleic acid-based detection methods such as Nucleic Acid Spot Hybridisation (NASH), Polymerase Chain Reaction (PCR, RT-PCR, real-time PCR), or more recently Loop-mediated isothermal Amplification (LAMP) (Boonham et al., 2014). Using these conventional direct detection methods to test for plant viruses requires *a priori* knowledge of the pathogens likely to be associated with a given host species allowing samples to be subjected to a panel of tests

giving a series of binary results (‘Positive’ or ‘Negative’) (Adams et al., 2018e, Maree et al., 2018). Traditionally non-targeted methods have relied on the visualisation of the presence of a virus either through symptom observation on a range of test plants following inoculation by grafting or through rubbing infected sap onto foliage with addition of an abrasive powder (Bioassay) (Verhoeven & Roenhorst, 2003, Roenhorst et al., 2013, Legrand, 2015), or through observation of viral particles by Transmission Electron Microscopy (TEM). These methods offer non-specific approaches for detection of viruses, and may give an indication of the identity of the virus detected, such as by symptom expression on a set of indicator hosts (Roenhorst et al., 2018), or particle morphology under a TEM (Brandes & Wetter, 1959). In some cases there have been reports of using non-target methods such as these for diagnosis (Zechmann & Zellnig, 2009). However, the application of these techniques and the interpretation of results relies on a high degree of experiential skill and knowledge. Additionally, due to the time and facility requirements to perform an individual test, these methods are not conducive to high-volume routine testing applications. Recently the development of High-Throughput Sequencing techniques (HTS), also known as Next Generation Sequencing (NGS) has offered the advantages of non-target detection with the ability to identify a given sequence to species level in a single test (Roossinck et al., 2015). Since the first reports of HTS being used for plant virus detection (Adams et al., 2009, Al Rwahnih et al., 2009, Kreuze et al., 2009) the technique has been developed beyond a research support tool into a tool for frontline virus diagnostics (Adams et al., 2018e, Maree et al., 2018). The technique has had the impact of accelerating the speed and frequency at which previously unknown viruses can be detected and their nucleic acid sequences characterised. The impact of these diagnostic developments is now evident in the publication record, as discussed by Fox and Mumford (2017). However, with this increased range of detection, there is the issue of how plant health authorities should react to findings of previously unknown plant pathogens (MacDiarmid et al., 2013, Massart et al., 2017, Adams et al., 2018e, Olmos et al., 2018).

A number of HTS studies have focussed on field-level virome studies (Coetzee et al., 2010, Alabi et al., 2015), or broad ecological studies (Roossinck et al., 2010). Many reports focus on elucidating the viruses present in single symptomatic samples with diseases of unknown aetiology (Roossinck et al., 2015). Despite many publications discussing the potential for the technology in plant health, including the implications and applications of the technology (MacDiarmid et al., 2013, Mumford et al., 2016, Adams et al., 2018e, Maree et al., 2018), or for handling the outcomes of diagnostic testing (Massart et al., 2017), there are still relatively few reports on the practical application of the technology in a plant health/biosecurity setting. However, the limited number of plant health case studies do demonstrate the application of the technology as part of a broader general diagnostic workflow in supporting other conventional diagnostic approaches (Fox et al., 2016a, Fox et al., 2016b, Skelton et al., 2018a, Skelton et al., 2018b, Fox et al., 2019). The other main areas of application are screening asymptomatic material, either in germplasm collections or as part of certification schemes (Maree et al., 2018). There are several hurdles to this technology being adopted for routine use. Whilst cost issues are being addressed through novel applications of long standing approaches such as pooling samples (Verdin et al., 2017), the practical considerations of experimental design, sampling and interpretation of results have not been addressed to the same extent. Additional skills and knowledge are also required to turn this technology into a meaningful tool in plant health, such as technology validation, virus characterisation, epidemiological transmission studies, and causation theory, all of which are necessary to support risk assessment for previously unknown pathogens (Adams et al., 2018e, Maree et al., 2018).

The publications presented in this thesis explore the use of diagnostic technologies in support of plant health virology including virus discovery, experimental design, sampling, result interpretation and assay validation. Chapter 2 (Fox & Mumford, 2017) discusses the drivers behind first detections of novel viral pathogens in the United Kingdom over the last 35 years, presenting data linking changes in research focus, plant trade, and diagnostic technologies to the number and type of detections. Chapter 3 (Adams et al.,

2013) details the use of HTS to identify the causal agents of Maize Lethal Necrosis, an emerging serious threat to food security in East Africa. Chapter 4 (Adams et al., 2014b) describes an investigation into a necrotic root symptom in carrots. It describes an experimental design linking conventional molecular diagnostics with HTS data to infer the causal agent of disease, where a demonstration of Koch's postulates was not feasible. This study detected four new virus species, including two species from a new genus. Chapter 5 (Fox et al., 2005) compares two methods for potato virus diagnostics over a time course and presents the advantages and disadvantages of each method. This study was the first to compare the advantages of a direct molecular analysis with a serological method. Chapter 6 (Fox et al., 2015) details the first use of HTS to simultaneously detect a complex of viruses and viroids from true seed. The study was a first in the published record to comparatively assess HTS and real-time RT-PCR. Chapter 7 (Fox et al., 2017a) reports a virus epidemiology study looking at transmission. This study identified previously unreported aphid vectors of potato virus Y (PVY; *Potyvirus*) and potato virus A (PVA; *Potyvirus*), as well as generating a first published relative transmission index for PVA. This thesis aims to discuss how these different elements link together in support of novel virus discovery through to plant health risk assessment and how the application of this work has contributed to advancing the field of plant health virology.

1.2 Discussion

1.2.1 Context of new findings

The development and application of new diagnostic technologies in support of plant health virology has been a major driving force in monitoring the spread of emerging plant viruses and in facilitating the discovery of previously undescribed pathogens. Chapter 2 is a review of first virus detections and novel discoveries in the UK, covering the last 35 years (Fox & Mumford, 2017). Discussing the drivers behind first detections at the national level and findings of novel viral pathogens, the review presents data on the number and type of detections in the UK. There are few comprehensive reviews of pest detections and interceptions at the national level, yet these data underpin the basis of quarantine regulations and plant health actions (Shivas et al., 2006, Jones & Baker, 2007). Often such reviews focus on these pathogens being new introductions to geographic regions but overlook the underlying drivers of discoveries. Whilst some of these will be recent introductions, Fox and Mumford (2017) also discuss the drivers of these detections, linking changes in research focus, plant trade, and the impact of improved diagnostic technologies to the patterns of novel findings and first detections for the UK. For instance improved diagnostics, combining HTS-based screening integrated with conventional methods, has led to the discovery and further investigation of multiple novel viruses in a broad range of plant species (Roossinck et al., 2015). Most recently these drivers also include low volume unregulated internet trade in plant species which may harbour pathogens which pose risk to cultivated staple crops such as a suite of novel viruses detected in *Ullucus tuberosus* (Fox et al., 2019). Although seemingly novel, these viruses were considered to be of regulatory significance due their genetic and serological similarity to quarantine viruses, and likely origin in South America. Additionally, this case study also highlights the potential similarity between the ‘novel’ viruses detected through sequencing and those viruses previously described from the host by either biological or serological characterisation.

The gap between virus discovery and demonstrating the plant health implications of these pathogen candidates is now a serious barrier to the routine application of HTS technology in regulatory plant health (MacDiarmid et al., 2013, Massart et al., 2017, Adams et al., 2018e, Maree et al., 2018, Olmos et al., 2018).

1.2.2 Developing high throughput sequencing for plant virology

On discovering a novel pathogen there are essential data that must be collected to support the risk assessment of that pathogen, these data can also support decisions on actions to contain or eradicate the pathogen if necessary. These data include taxonomic placement, epidemiology including information on transmission pathways, host range, and not least, assessing the causal relationship to disease and the potential impact of the pathogen (Massart et al., 2017, Adams et al., 2018e, Olmos et al., 2018).

Chapters 3 and 4 describe the detection of characterised and previously uncharacterised viral pathogens by High-Throughput Sequencing from field grown samples affected by diseases of unknown aetiology. Wangai et al. (2012) reported the presence of Maize Lethal Necrosis disease in Kenya, similar to the previously reported corn lethal necrosis, it is a synergistic disease complex resulting from simultaneous infection by maize chlorotic mottle virus (MCMV, genus *Tymovirus*) and a potyvirus (Niblett & Claflin, 1978). Simultaneous work reported by Adams et al. (2013) (Chapter 3) demonstrated that the disease was caused by divergent isolates of previously characterised viruses and that conventional diagnostic approaches could be unreliable for the detection of these pathogens. The disease was shown to be rapidly emerging as a food security issue throughout East and Central Africa (Adams et al., 2014a, Lukanda et al., 2014, Mahuku et al., 2015a, Mahuku et al., 2015b, Fentahun et al., 2017). This discovery has also facilitated further research aimed at controlling the disease such as gaining a better understanding of the

aetiology of the disease (Stewart et al., 2017), and screening maize breeding lines for disease resistance (Beyene et al., 2017).

This integrated diagnostic approach was also applied in other crops with diseases of unknown aetiology. For at least ten years, a necrotic root symptom had been evident in carrot crops in the UK. Anecdotal evidence from growers and horticultural consultants, using approaches based on likely candidates and available diagnostics, failed to reveal a causal relationship between the viruses that were detected and the symptom. Using HTS, Adams et al. (2014b) revealed multiple potential disease causing candidates including several novel viruses and previously characterised pathogenic viruses. The study aimed to utilize experimental design, conventional and next-generation diagnostic technology and statistical analysis to address alternative approaches to inferring causal association from any such complex infections, where experimental demonstration may not be feasible. This study indicated a potential causal agent of the observed symptom in carrot yellow leaf virus (CYLV; *Closterovirus*) and also revealed multiple hitherto undescribed viruses in UK carrots as incidental findings. Among the previously undescribed viruses was a tentative novel virus, carrot closterovirus-1, closely related to *Carrot yellow leaf virus* which appeared to be a possible disease causing substitute species, present in the few necrotic carrots where CYLV was absent. Two novel betaflexiviruses were revealed from a new genus, *Chordovirus*, namely carrot ch virus-1 and carrot ch virus-2. Additionally, a novel virus from the genus *Torradovirus,* carrot torradovirus-1 (CaTV-1)*,* was also described. Previously described members of the genus *Torradovirus* had been largely found infecting tomato crops (Verbeek et al., 2007, Verbeek et al., 2008, Verbeek et al., 2010a, van der Vlugt et al., 2015), and these tomato-infecting torradoviruses were shown to be transmitted by whiteflies (Verbeek et al., 2014b, Amari et al., 2017). Among the non-tomato-infecting torradoviruses, three appeared to form a distinct clade *Lettuce necrotic leaf curl virus, Motherwort yellow mottle virus*, and *Carrot torrado virus-1* (Adams et al., 2014b, Verbeek et al., 2014a, Seo et al., 2015, van der Vlugt et al., 2015, Rozado-Aguirre et al., 2017a). CaTV-1 was further characterised and real-time PCR diagnostic assays were developed (Rozado-Aguirre et al., 2016, Rozado-

Aguirre et al., 2017a). Field distribution data indicated that infected plants were present at high incidence which was greater towards field margins and hedgerows, implicating the potential involvement of an invertebrate vector introducing the virus into the crop (Fox et al., 2017b). Given the absence of reports of whiteflies from carrot crops in the UK it was thought to be unlikely that this virus was being transmitted by whiteflies and it was demonstrated that it was transmitted by aphids (Rozado-Aguirre et al., 2016). Subsequent work on LNLCV also implicated aphid vectors in the epidemiology of this virus, suggesting a clade of non-tomato-infecting torradoviruses with aphid vectors (Verbeek et al., 2017). CaTV-1 was later shown to be present in carrot crops in mainland Europe (Rozado-Aguirre et al., 2017b), in an uncultivated species in Greece (Lotos et al., 2018), in a perennial apiaceous herb in Japan (Tokuda et al., 2019), and in *Apium graveolens* in Germany (Gaafar & Ziebell, 2019), however the impact of this virus remains unclear.

Because of work such as that detailed above, HTS is now routinely applied in the laboratories at Fera Science Ltd, York, UK. This has led to multiple first detections and disease notes being published using the methods described (Harju et al., 2012, Fox et al., 2016a, Fox et al., 2016b, Reeder et al., 2017, Skelton et al., 2018a, Skelton et al., 2018b). Additionally, applying an integrated diagnostics approach informed by HTS in a regulatory plant health setting led to the detection and discovery of multiple novel viruses in the species *Ullucus tuberosus.* HTS was used to provide definitive diagnosis where conventional targeted methods such as ELISA, RT-PCR, and real-time RT-PCR could not, due to their specificity (Fox et al., 2019). The HTS data were also used to support initial outbreak risk assessments for the novel viruses. This included viruses considered to be of Andean origin with the potential to affect solanaceous species, which led directly to a change in EU legislation. *U. tuberosus* has consequently been added to the list of species with prohibited entry into the EU territory (Commission Implementing Regulation (EU) 2018/2019).

1.2.3 Understanding the aetiology of disease

1.2.3.1 Koch's postulates

Given the rate of novel virus discovery facilitated by HTS, there is a renewed imperative to explore the inference of causal relationships between detections and disease, separating out the causal agents from the commensal organisms. Definitively linking a novel candidate pathogen with an observed symptom of disease requires extensive biological characterisation (MacDiarmid et al., 2013, Massart et al., 2017, Adams et al., 2018e). In plant pathology the accepted paradigm for causation is the experimental demonstration of Koch's postulates following isolation of the putative pathogenic agent (Rivers, 1937, Evans, 1976). These causation criteria, as expressed by Robert Koch, were (A) The pathogen occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease. (B) The pathogen occurs in no other disease as a fortuitous and non-pathogenic parasite. (C) After being isolated from the infected host and repeatedly grown in pure culture, the pathogen can induce the disease when introduced to a previously unaffected host (adapted from Rivers (1937) translation of Koch's original work). An adaptation of Koch's postulates, largely focussing on aspect (C) has been adopted as the gold standard in plant pathology.

1.2.3.2 The limitations of Koch's postulates in plant virology

Koch's postulates have been widely reviewed, discussed and adapted in the light of subsequent scientific developments in biomedical sciences and occupational medicine (Bradford Hill, 1965, Evans, 1976, Fredericks & Relman, 1996). However, in plant pathology these postulates dogmatically remain the prevailing principles for inferring causation. The postulates are based on the one pathogen-one disease paradigm of infection biology and are inadequate in cases of diseases with polymicrobial causes such as Acute Oak Decline (Denman et al., 2017). Even as early as 1937 the limitations of Koch's postulates were recognised in the then emerging field of virology (Rivers, 1937). For example, at the gross scale, plants have a limited range of reactions

to viral infections, and more than one virus species may induce similar disease symptoms on a given host e.g. the spraing inducing viruses of potato (*Solanum tuberosum*) potato mop-top virus and tobacco rattle virus (Mumford et al., 2000). Conversely, the same virus species may induce a diverse range of observable symptoms, or may infect asymptomatically on a given host species due to differences in virus isolate, cultivar, time after infection, and environmental conditions, providing a significant challenge in satisfying Koch's first two postulates. As obligate pathogens, viruses cannot be grown outside of susceptible hosts, and even if a virus could be isolated from an infected host into a pure culture, growing this in an alternate host or in tissue culture host may not adequately satisfy Koch's third postulate (Rivers, 1937). An additional complication arises because the majority of plant-affecting viruses are RNA based, with poor replication fidelity leading to them existing as quasispecies, or clouds of mutant genomes (Eigen et al., 1989, Domingo, 2002). This phenomenon, which is thought to confer fitness to adapt to changing evolutionary pressures, also has the consequence that obtaining a 'pure' population in *sensu stricto* will not be achievable. Simultaneous co-infections of viruses, or multiple virus strains, in field-grown plants are not uncommon (Adams et al., 2014a, Adams et al., 2014b, Skelton et al., 2018a, Skelton et al., 2018b, Fox et al., 2019). Separating out multiple viruses to demonstrate whether a disease is due to a single component of a polyspecies infection, or the result of interactions between multiple viruses in complex, is time consuming and technically challenging. Some viruses may only be transmissible through a vector, or may require a helper virus, or encapsidation to facilitate transmission, such as in the case of carrot necrotic dieback virus (formerly Anthriscus strain of *Parsnip yellow fleck virus*) and carrot mottle virus in the carrot motley dwarf virus complex. Additionally, there may be multiple broad host range viruses present, which provide significant challenges when trying to isolate novel viruses from complex infections, as this would normally be achieved through single lesion isolation or through use of differential hosts. One proposed universal solution to support virus characterisation is the broader use of infectious clones in such studies (Massart et al., 2017), however, creating infectious clones requires time and resource. In cases

where multiple novel viruses are detected in single or bulked samples, e.g. the case of viruses of *Ullucus tuberosus* (Fox et al., 2019) or carrot root necrosis (Adams et al., 2014b), or where a study generates many novel viral pathogen candidates, such as the Kenyan maize virome (Adams et al., 2018e), the combination of prioritising viruses for further study combined with the need for rapid risk assessment renders this approach unwieldy in a plant health setting.

Although these limitations of Koch's postulates have been previously recognised (Massart et al., 2017), the suitability of alternate approaches for determining causal relationships of disease have not been discussed with respect to plant virology (Adams et al., 2018e). The cases presented in Chapters 3 and 4 generated a combination of basic epidemiological observations, biological characterisation, and extensive sequence data on the presence of viruses in affected and unaffected hosts. Although both studies were based upon HTS, they also generated real-time PCR and PCR primers to facilitate further high throughput cost effective studies of virus distribution and impact. Given these factors and the limited applicability of Koch's postulates, alternate approaches for inferring causation would be desirable. Rivers (1937) gave two points for considering causation in a virus-disease relationship: (a) A specific virus must be found associated with a disease with a degree of regularity and (b) The virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation. In a case such as Pansy Mottle Syndrome with a reported association with the presence of viola white distortion-associated virus (Ciuffo et al., 2014), further biological work would still be needed to confirm a causal link due to the relatively poor incidence and correlation between virus and disease (Fox et al., 2016b). Rivers' first criteria is therefore too subjective for effective application. Whilst a disease such as carrot root necrosis could be shown to be regularly associated with carrot yellow leaf virus (CYLV) through diagnostic supported aetiological observation (Adams et al., 2014b), the proportion of asymptomatic roots infected with CYLV suggests a background of asymptomatic-infected individuals in the population and does not allow for Rivers' second consideration to be satisfied. However, this

second consideration may, in effect, be interpreted as the same requirement for an experimental demonstration of causation proposed by Koch.

1.2.3.3 Alternate approaches to causal inference

In 1965, Sir Austin Bradford Hill gave the President's address to the Section of Occupational Medicine, at the Royal Society of Medicine (Bradford Hill, 1965). As a medical statistician Bradford Hill's address was focused on the largely abiotic environmental factors found in Occupational Medicine. The nine principles discussed, now termed 'The Bradford Hill criteria' (Table 1), have become widely referenced in support of determining causal associations with disease.

Table 1. The Bradford-Hill criteria for inferring causation from epidemiological observation (Bradford Hill, 1965)

Criteria	**Consideration**
Strength	How strongly correlated is the association between putative cause and disease?
Consistency	Is the same finding observed in different populations in different places/times/etc?
Specificity	Is the effect seen in a specific population without any other likely explanation?
Temporality	Does exposure come before effect?
Biological Gradient	Is there an observed dose response?
Plausibility	Is there a plausible relationship/mechanism between factor and effect?
Coherence	Is the causal association compatible with present knowledge of the disease?
Experiment	Can the relationship be investigated by experimentation?
Analogy	Does the relationship between factor conform to a previously described relationship?

The criteria include considerations of the strength, consistency, and specificity of the relationship between symptom observation and the presence of a putative causal agent. The criteria take into account factors relating to symptom development such as a temporal relationship or biological gradient, i.e. is the pathogen candidate present before symptoms are observed and is there a dose response? It also considers whether a relationship can be considered valid where an analogous relationship is observed elsewhere. Hill also included considerations gained through experimental investigations, such as the coherence and plausibility of a causal relationship, ensuring that epidemiological observation aligns with experimental results and that there is a likely mechanistic relationship that can be demonstrated. Within the criteria there is also the consideration of demonstrating the relationship through experimental investigation, either through a positive demonstration (i.e. Koch's postulates) or through a reduction in symptom development by exclusion of the putative causal agent. Bradford Hill (1965) notes that this is where the strongest evidence of a causal relationship is likely to arise. These criteria were not intended to give an absolute demonstration of causation, but instead to provide a framework against which epidemiological data could be weighed in determining a causal association. These criteria have also been reviewed in light of the use of data integration in molecular epidemiology (Fedak et al., 2015). They are adaptable and often cited in biomedicine. Despite them having been previously used to indicate potential causal relationships in virology, such as associating human papilloma viruses (Genus *Betapapillomavirus*) with oropharyngeal carcinoma (Walvik et al., 2016), they have not yet been applied within a plant virology study. Latterly, Fredericks and Relman (1996) proposed seven points for considering molecular evidence for determining causal relationships between pathogen and disease using nucleotide sequence based detection methods (Table 2). The considerations broadly align with those of Bradford Hill (1965).

Fredericks and Relman (1996) proposed that sequence of the putative pathogen should be present in 'most' cases of disease and associated with organs with pathology, that fewer or no sequences should be present in asymptomatic organs, and that tissue sequence correlates should be

investigated at the cellular level, these broadly align with Bradford-Hill's criteria of specificity, strength and consistency of relationship. The consideration that the amount of sequence should decrease with resolution of disease, and conversely increase with relapse, and that the putative pathogen should be present before disease, are consistent with temporality and biological gradient (dose response).

Table 2. Criteria for inferring causation from molecular (nucleic acid sequence based) data after Fredericks and Relman (1996)

i.	Nucleic acid sequence of the putative pathogen should be present in 'most' cases of an infectious disease (and should be associated with organs showing pathology)
ii.	Fewer/no sequences should be detected from hosts or organs without pathology
iii.	When disease is resolved sequence should decrease, and increase with relapse
iv.	Detection before disease, or a dose response is more likely to indicate a causal relationship
v.	The nature of the putative pathogen inferred from the sequence should be consistent with characteristics of that group of organisms
vi.	Tissue sequence correlates should be sought at the cellular level
vii.	Sequence based forms of evidence should be reproducible

Fredericks and Relman also included considerations of analogy, plausibility and coherence, in that they concluded that the nature of the putative pathogen inferred from the presence of sequence should be consistent with the characteristics of that group of organisms. Seeking tissue/sequence correlates at a cellular level could be achieved through either in situ hybridisation assays or by observation of cellular changes, such as cytoplasmic pinwheel inclusions, although these may be induced by other factors (Wilson et al., 1976). Additionally, the requirement that sequence-based forms of evidence should be reproducible strongly implies a need for gathering such data through experimentation. Adams et al. (2013) discovered that maize plants inoculated

with sap from maize lethal necrosis (MLN) symptomatic plants infected with maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) developed necrotic streaking, although only MCMV was found to be present in the inoculated test plants. This cannot be considered as a successful demonstration of Koch's postulates. Despite this, both existing reports from outside Africa and subsequent reports of the disease distribution associated with both viruses suggested a strong, consistent, coherent, plausible causal relationship (Niblett & Claflin, 1978, Xie et al., 2011, Adams et al., 2014a, Lukanda et al., 2014, Mahuku et al., 2015a, Mahuku et al., 2015b, Fentahun et al., 2017). However, the specificity of this relationship remains in question with a range of viruses potentially inducing symptoms that are consistent with MLN (Adams et al., 2017).

1.2.3.4 Applying statistical approaches for causal inference

The other approach often taken to infer causal relationships is the application of Bayesian probability. These approaches have been applied in many disciplines where there is a need to draw causal inferences from large datasets. The main application of Bayesian probability in plant pathology is in calculating the likelihood of genetic relationships during the construction of phylogenetic trees. However, using a biostatistical approach can also be of use where other experimental demonstrations of causation cannot be readily achieved. This approach was taken in investigating carrot root necrosis (Adams et al., 2014b). As multiple potential causal agents were present in both symptomatic and asymptomatic samples, it was apparent that symptom development was not a consistent cause-effect relationship. The statistical analysis indicated that removing carrot yellow leaf virus from the pathosystem would reduce the presence of necrotic carrot roots by 96%, and no other virus present in the samples was significantly associated with the presence of necrosis. This gives weight to the strength of the association, despite the presence of large numbers of virus positive asymptomatic roots. Similarly, as with the situation observed with MLN, there remains the question of the specificity of the relationship. In the sequence analysis one symptomatic root was found to contain a novel closterovirus (tentatively named carrot

closterovirus-1) rather than carrot yellow leaf virus. However, as both viruses are closely related, this could be an analogous causal relationship and further work needs to be carried out to confirm this.

A statistical approach must be applied with caution and preferably not in isolation. Care should be taken to ensure that the experiment is appropriately designed and controlled, and associated observations are made on the incidence, impact and epidemiology of the disease under investigation. Pansy Mottle Syndrome is another malady of unknown aetiology affecting cultivated *Viola* hybrids (Pansies), which has been recognised since the 1960's (Fox et al., 2016b). Symptoms include deformation of leaves and white 'bleaching' in patches of foliage rendering plants unfit for sale (Figure 2).

Figure 2. Bleaching and leaf deformation, a symptom of Pansy Mottle Syndrome, associated with infection by viola white distortion associated virus.

Symptom development was unpredictable and often transient, but has been anecdotally linked to periods of high temperature and light intensity. Ciuffo et al. (2014) reported the presence of a novel virus tentatively named viola white distortion associated virus (VWDaV, Genus *Ilarvirus*) in *Viola x wittrockiana.* Similarly to the carrot situation described above (Adams et al., 2014b), a high number of asymptomatic infected individuals combined with unknown factors influencing symptom development provided significant challenges to making

inferences on causal associations between the pathogen candidate and disease. An attempt was made to apply an analogous approach to that used in the carrot work to investigate this issue (Fox et al., 2016b). However due to the unpredictable nature of symptom development adequate numbers of affected and unaffected plants could not be obtained from a single crop or even nursery, meaning that the samples tested were from multiple nurseries and a range of varieties. Samples were tested in three groups representing two symptom types and an asymptomatic control. A high proportion of pansies tested (63.5%-73.3%) were positive for the virus in both symptomatic and apparently healthy (asymptomatic) sample groups. These data suggest that the virus is widespread in pansy germplasm, and whilst this does not exclude the virus as a component of Pansy Mottle Syndrome there are other drivers of symptom expression which remain undetermined.

The examples discussed above demonstrate how improved diagnostic technologies, such as HTS, can be applied in frontline plant health laboratories to support investigations into causal relationships where traditional approaches are fruitless. The experimental demonstration of causation should and will remain the gold standard. However, given this is not always possible, or may be undesirable due to the time taken to gather appropriate data, an approach utilising epidemiological observations, supported by statistical analysis, based on rigorous observation and careful experimental design can give valid insights into causal relationships.

1.2.3.5 An integrated approach for plant virology

From considering the frameworks suggested by previous authors (Rivers, 1937, Bradford Hill, 1965, Evans, 1976, Fredericks & Relman, 1996, Fedak et al., 2015) and the more recent reports of investigating diseases of unknown aetiology (Adams et al., 2014b, Ciuffo et al., 2014, Fox et al., 2016b, Denman et al., 2017), integrating these approaches allows a hierarchical set of causation criteria to be formulated (Table 3). Determination of causation can be simplified to four key considerations encompassing the multiple criteria previously reported. These are experimental evidence, strength of relationship, consistency of relationship, and the dual consideration of coherence and

plausibility. The application of these considerations should not be rigid and could be approached either serially or sequentially. They should be applied on a case by case basis and are intended to encourage a rigorous approach to considering causation.

Table 3. A simplified hierarchical approach for considering a causal relationship in plant virology.

Criteria	**Suggested approaches**
Experiment	An isolate should be inoculated into an uninfected host and observed for symptom development, thus satisfying Koch's 3rd postulate and Bradford-Hill's Experiment criteria.
Strength	This should be based on field/glasshouse observation. Experimental design should as a minimum include HTS and statistical analysis of affected and symptomless individuals, accounting for polymicrobial effects and latent infections.
Consistency	This can be considered using the same approach as for Strength above but should be repeated at multiple geographic locations and/or at different times.
Coherence and Plausibility	Are there any confounding factors in these data, e.g. symptomatic individuals without evidence of infection or does symptom appear before infection? Are there similar effects reported in other pathosystems to support the conclusions? This could be the same, or related viruses in the same, or related hosts.

Experimental demonstration should remain the primary consideration, this could be at the macro-level, or could be on specific plant parts e.g. roots or fruit, or could be at the cellular level, satisfying Fredericks and Relman's criteria (ii) and (vi). Strength of relationship should also include a measure of the specificity of the causal relationship, but this should not be an absolute. Strength of relationship in itself does not indicate causation, but instead gives a sound basis for further investigations. Consistency of relationship could be considered to satisfy the 'repeatability' criteria of Fredericks and Relman (1996) with respect to strength of the relationship between cause and effect. If

appropriate supporting metadata are also collected, such as field conditions, agronomy, prevailing environmental conditions etc. Consistency data should also indicate if there are contributing abiotic factors, cultivar influences, or the impact of polyspecies or substitute species. This consistency also encompasses Bradford Hill's (1965) 'specificity'. Considerations of coherence and plausibility become a 'catch all' acting as a check on the data gathered in support of strength and consistency measures outlined above.

1.2.4 Validation of methods

The ability to determine whether a given test result is reliable, and whether a laboratory is competent to conduct such testing, is essential. Measurement of whether a test is 'fit for purpose' is achieved by determining the performance characteristics of a test (validation) and monitored through ongoing quality control measures to ensure a test is performing within predetermined parameters. Basic quality control measures, such as the inclusion of appropriate positive and negative controls, can be used to give an ongoing measure that a test is performing as expected. In some methods, such as ELISA, these controls are also essential in ascertaining a positive sample result with reference to a threshold calculated against a known negative control result (Clark & Adams, 1977, Sutula et al., 1986). In other methods a more complex array of controls may be needed as described by Chabirand et al. (2016).

1.2.4.1 Validation in plant health laboratories

The need for effective validation in plant health diagnostic laboratories has increased due to a focus on accreditation to international standards such as ISO 17025:2005 (ISO/IEC, 2005) being used to demonstrate the competence of a laboratory to perform a test. Accreditation may be gained at either the test level (using a method for detecting a specific target on a specified host matrix), known as 'fixed scope', or where a generic method is used, such as real-time PCR for nucleic acid-based detection, it may be attained at the method level,

termed ‘flexible scope’. To guide plant health laboratories in validating the array of tests used to an acceptable level, the European Plant Protection Organisation (EPPO) has produced technical standards on basic quality management and validating tests (OEPP/EPPO, 2018a, OEPP/EPPO, 2018b). However, prior to these standards being published, laboratories carried out validation in a more ad-hoc manner. Where there has been a demonstrable need to improve the reliability or performance of an existing test this was achieved through comparative method performance studies. An example where this has been carried out over many years is in post-harvest testing of potato tubers. Field certification of seed potato crops based on visual observation can be unreliable as current season (primary) infections cannot be readily observed. Additionally, due to low virus titre in the case of late season primary infection (infection during the growing season) and potato-infecting potyviruses being difficult to detect following periods of tuber storage (De Bokx & Cuperus, 1987, Barker et al., 1993), direct detection using serological methods (e.g. ELISA) is unreliable. Therefore, the current global standard test was, and still is, a forced grow-out where tuber dormancy is broken through either the use of gibberellic acid (Fox et al., 2005), ‘Rindite’ (ethylene chlorhydrin – ethylene dichloride – carbon tetrachloride 7 : 3 : 1) (Varga & Ferenczy, 1956, Vetten et al., 1983), or through natural sprouting (Hill & Jackson, 1984). The resulting grown-out plantlets are then tested for the presence of viruses using serological methods, such as ELISA. However, this method is time consuming as it can take 4-6 weeks to complete testing, and plants have to be grown out in a glasshouse, making large scale testing by this method resource intensive. Multiple studies attempted to determine more reliable and sensitive methods for direct detection of potato-infecting potyviruses including Digoxigenin-labelled probes (DIG-probes) (Singh & Singh, 1995). In the early 2000’s an improved PCR technique, real-time PCR was developed for the detection of a range of potato viruses (Boonham et al., 2000, Mumford et al., 2000) and later published as a laboratory protocol (Boonham et al., 2009). Fox et al. (2005) (Chapter 5) aimed to assess the reliability of direct detection of PVY by real-time RT-PCR and ELISA, comparing it to the traditional ELISA-supported growing out test. Unlike the

previous work of Barker et al. (1993) the study aimed to give a true time course of detection throughout the storage period, rather than an end point. The study also gave a measure against an 'overall' positive result for each tuber test by combining results from each test method with those obtained by testing the remaining tuber, with the aim of determining the false negative rate for each method. The study demonstrated the reliability of direct detection by nucleic acid-based methods throughout the post-harvest storage period, providing supporting evidence for the broad adoption of real-time PCR.

This approach of testing method performance against a previously validated method remains an integral part of the validation process (OEPP/EPPO, 2018b). However, in 2009 the publication of this standard introduced set performance characteristics such as determining the sensitivity (limit of detection; LoD), specificity (range of detection), also testing to determine the robustness of a method including selectivity (do cultivar or host matrix influence upon the test), repeatability (is the test repeatable at LoD), and reproducibility (does the test work on different brands of machine, on different days, with different operators). The standard also introduced annexes giving criteria to allow these characteristics to be assessed for the breadth of test methods applied across all biological disciplines (Chabirand et al., 2016, OEPP/EPPO, 2018b). However, apart from morphological identification (microscopy), the approaches listed apply to targeted methods, and require the scope of a test to be specified for a given method to detect a pathogen target in a specifc host and matrix. The validation of a universal non-target method, such as HTS, therefore presents a challenge in defining the scope for validation, and in effect cannot be fully validated for the detection of 'unknown' pathogens.

1.2.4.2 Validation of HTS

As part of an approach to understand how HTS could be validated broadly in line with the EPPO standard, Fox et al. (2015) (Chapter 6) investigated the application of sequencing for the simultaneous detection of viruses and viroids from tomato seeds. Solanaceae-infecting pospiviroids present a distinct challenge to plant health diagnostic laboratories. Viroids do not code for

proteins, so cannot be detected using serological approaches, they are robust due to having a complimentary secondary structure, they are relatively thermally stable and can survive outside of a host for extended periods (Mackie et al., 2015). Viroids are reported to be seed-transmissible pathogens, and there have been several outbreaks in commercial tomato and pepper crops associated with seed transmission, although the reported rates of seed transmission are variable and the importance of seed as a transmission pathway remains unquantified (EFSA, 2011, Chambers et al., 2013, Van Brunschot et al., 2014, Constable et al., 2019). This uncertainty of the risk of solanaceous seed as a transmission pathway, combined with the high volume of internationally traded seed has resulted in many countries adopting diagnostic testing for an increasing range of pospiviroids from consignments of solanaceous seeds (mainly tomato and pepper) entering their territories. For instance, to export a consignment of tomato seeds to Australia requires a test of 20,000 seeds, for a range of nine tomato-affecting pospiviroids (Constable et al., 2019). Therefore, there was a demonstrable need to investigate a universal detection method which could offer the sensitivity of molecular detection allied to a broad multiplex detection capability. The aim of the study described by Fox et al. (2015) was to investigate the relative sensitivity of HTS for detecting two pospiviroids (potato spindle tuber viroid and columnea latent viroid), and pepino mosaic virus, using an rRNA-depleted total RNA sequencing strategy with the current laboratory standard protocol. The study used a serial dilution approach, diluting known infected seeds in supposedly uninfected seed which was commercially purchased and imported to the UK under a plant passport. The study demonstrated that HTS could detect the two pospiviroids in the seed samples, despite there being a loss of sensitivity by comparison to the real-time RT-PCR method. However, the study also showed that the supposedly healthy seed was contaminated with multiple tomato-infecting viruses as the analysis indicated that the HTS approach had simultaneously detected pepino mosaic virus, tobacco mosaic virus, cucumber mosaic virus, tomato bushy stunt virus, potato leafroll virus and a novel member of the genus *Cavemovirus* from both the serial dilution samples and the ‘uncontaminated’ control sample. This was the first report to use HTS to

simultaneously detect a range of viruses and viroids from true seed of tomato (*Solanum lycopersicum*), demonstrating the potential for this method for application in import screening.

Validation of HTS has been previously discussed for clinical applications, and these findings may prove useful as guidance for plant pathology applications to indicate possible approaches to validation (Mattocks et al., 2010, Frampton et al., 2013, McCourt et al., 2013, Salto-Tellez & Gonzalez de Castro, 2014). Essential validation factors have been investigated such as defining the minimum depth of sequencing for reliable detection of viruses (Visser et al., 2016), and assessing the relative merits of different sequencing strategies across a range of virus taxa (Pecman et al., 2017). Despite these advances, HTS has still not been fully validated for use in plant virology. The challenges, and costs, which have inhibited broad uptake of the method, allied to the plant health concerns related to detecting previously unknown viruses have restricted progress in this area. Ultimately, the existing standard frameworks, such as EPPO PM7/98 (OEPP/EPPO, 2018b), can be readily applied for validating HTS as a 'megaplex' diagnostic platform for previously characterised pathogens. As validation cannot be achieved for 'unknowns', these detections will require further confirmation preferably employing a different biological principal. However, as plant health virus diagnostics is a multiphasic process, the principal of confirmation testing using a separate test should continue to be used to ensure a system-level validation of novel detections by HTS (Roenhorst et al., 2018).

1.2.5 The vector specificity of some aphid-borne viruses

1.2.5.1 The background

The dissemination and establishment of a virus into a new geographic region is intrinsically linked to the movement of infected host plants and/or viruliferous vectors. Once an infected host has been planted, usually in the form of a propagation cutting, a bulb or seed tuber, the main plant health risk then comes

from onward transmission of the virus to uninfected hosts. Beyond the viruses considered to be of quarantine importance such as those specifically listed in the EC plant health directive (2000/29/EC), there are a large number of viruses which are already present in the UK which cause yield and quality impacts. Where these viruses are recognised to be disseminated through vegetative propagation their spread and consequent impact may be mitigated through plant health propagation schemes. Such schemes are used to ensure high health input planting material for a range of vegetatively propagated crops including soft fruits (*Fragariae, Rubus and Ribes*), and top fruits (*Malus*, and *Pyrus*). The most common of these schemes are for the production of seed potatoes. Within seed potato certification schemes historically the virus most commonly detected was the persistently transmitted potato leaf roll virus (PLRV). However, from the 1990's the emergence of more effective chemical controls meant that non-persistently transmitted viruses, particularly the potyviruses including PVY, became more prominent (Pickup et al., 2009). This virus has a global distribution and in the UK it was noted that the incidence of potato virus A was markedly higher than the incidence in other seed potato producing countries, responsible for up to 22% of mosaic virus symptoms observed during official inspections in Scottish seed potatoes (Pickup et al., 2009, Pickup et al., 2010). This was considered to be partly due to the influence of varietal susceptibility. Investigations were also conducted over four years to examine the timing of transmission of this virus by comparison to strains of potato virus Y, to see whether there were potential differences in the suite of aphid vectors driving transmission of this virus (Pickup et al., 2010). However, this correlative approach required further supporting data to gain a better understanding of the dynamics of epidemics of PVY and PVA. Additionally to facilitate the development of prediction-based decision support for virus management based on vector monitoring (Northing, 2009), further work was needed to validate the relative transmission efficiencies (Relative Efficiency Factor; REF) of aphid vectors with emergent recombinant strains of PVY. Previous studies into relative transmission efficiencies had been conducted, showing that PVY was transmissible by at least 25 species of aphid, but these had employed a range of methods for acquiring viruliferous

aphids, and a variety of inoculation hosts, and consequently the outcomes of these REF studies were not directly comparable (see review by Lacomme et al. (2017)).

1.2.5.2 The general approach

One study comparing PVY^{O}, PVY^{N}, PVY^{NTN}, and $PVY^{N\text{-}Wilga}$ strains had used a single aphid clone as a reference control, combined with high numbers of individual aphids in each repetition, to allow for comparisons between relative transmission efficiencies for each aphid species with each virus strain (Verbeek et al., 2010b). To allow comparability with UK isolates and aphid clones the method was replicated by Fox et al. (2017a) (Chapter 7) utilising the same *Myzus persicae* control clone (Mp2). This study reported, for the first time, the willow-carrot aphid (*Cavariella aegopodii*) as a moderately efficient vector of PVY, and reported a first set of REFs for PVA, including first reports of *Aphis fabae, Metopolophium dirhodum, Sitobion avenae, Acyrthosiphon pisum* and *C. aegopodii* as vectors of PVA. Additionally, the study used a binomial (logit) model to examine inter-clone, and virus strain variation in transmission efficiency, demonstrating that the greatest influence was virus titre in the donor leaf. The REFs generated across these studies were then used to support the UK potato aphid monitoring programme (Northing, 2009, AHDB, 2019).

1.2.5.3 Applying the same methodology to other viruses

Following the discovery of a novel member of the genus *Torradovirus*, CaTV-1, infecting carrots (*Daucus carota*) in the UK (Adams et al., 2014b), epidemiological observations suggested that the virus was vectored by an insect (Fox et al., 2017b). The previously described torradoviruses had been demonstrated to be transmitted by whitefly vectors (Verbeek et al., 2014b, van der Vlugt et al., 2015, Amari et al., 2017). However, a lack of entomological records for whitefly on carrot in the UK, indicated these were unlikely to be the vector driving transmission of CaTV-1. Therefore, aphid transmission of CaTV-1 was studied using a method modified from previous studies (Verbeek et al., 2010b, Rozado-Aguirre et al., 2016, Fox et al., 2017a). This study demonstrated that both *C. aegopodii* and *M. persicae* were able to vector the

virus, the first torradovirus shown to be aphid-vectored (Rozado-Aguirre et al., 2016). *M. persicae* had not been widely recognised as a vector of carrot viruses. For instance, Waterhouse and Murant (1983) found that *M. persicae* could not transmit carrot red leaf virus (CtRLV; *Polerovirus*). However, a recent study indicated that, on some hosts at least, *M. persicae* could transmit CtRLV and consequently the other viruses of the carrot motley dwarf complex (Naseem et al., 2016). This information, combined with studies on other carrot viruses (Latham & Jones, 2004) suggest that *M. persicae* should be re-considered as a vector of carrot viruses in the UK. Additionally, studies on another non-tomato-infecting torradovirus, lettuce necrotic leaf curl virus (LNLCV), demonstrated transmission of the virus by the aphid species *Nasonovia ribisnigri*, and acquisition of the virus by *C. aegopodii* (Verbeek et al., 2014a, Verbeek et al., 2017).

1.3 Conclusions and Future prospects

The publications discussed here use an integrated diagnostics approach in plant virology to support a range of investigations, such as elucidating the causal agents of diseases of unknown aetiology, and also validation studies to demonstrate the performance characteristics of diagnostic methods. These papers also demonstrate a significant contribution to expanding the scientific knowledge of plant virology and to the development of novel diagnostic techniques into frontline applications. Over the last decade, high throughput sequencing has undoubtedly revolutionised plant virus diagnostics, revealing a new diversity of plant viruses and shedding light on hitherto unrecognised viruses such as those with a ‘persistent lifestyle’ (Roossinck, 2010). However, in terms of plant health virology, the fundamentals remain largely unchanged, on finding a new pathogen candidate studies need to be conducted to demonstrate the impact, incidence, distribution, and associated risk of the pathogen. During the period of writing this thesis there has also been the first reported use of HTS to resolve the identity of multiple novel viruses of plant health regulatory significance (Fox et al., 2019), an application of this technique only made possible from the previous HTS development work discussed in this thesis.

1.3.1.1 Closing the gaps

There remain applications of HTS in diagnostics which need to be further developed. For example, the potential of HTS methods to give an unbiased picture of the virus health status of a plant could greatly enhance current procedures in post-entry quarantine or certification of propagation material. These approaches could give a quantifiable measure of freedom from viral diseases, allowing plants to be traded internationally with a ‘metagenomic plant passport’ (Maree et al., 2018, Villamor et al., 2019). This would circumvent the current extensive time periods taken for post-entry quarantine, greatly reducing the costs of the process for both regulatory authorities in conducting the tests and for growers in having to delay commercial production. However, to get to a point of international acceptance by regulatory authorities

there needs to be a focus on validation of the technology for inclusion in diagnostic standards. The capacity for sequencing in bulk would see the 'per sample' cost reduce. One key factor which affects both the ability to bulk samples and to give confidence that the method can adequately detect total viral nucleic acids from a sample is to quantify the necessary minimum sequencing depth. There are few studies looking at this aspect of work (Visser et al., 2016, Pecman et al., 2017), more work is needed to elucidate minimum depth of sequence for different viral taxa or sample host matrices across different sequencing strategies and platforms. This could then allow HTS to be used as a generic technology, sequencing from 'whole field' samples in bulk to show presence or absence of pathogens in a cost-effective manner. Alternatively, this approach could be used to identify candidate pathogens from bulked samples, supported with follow-up testing by conventional methods to also give further data on incidence and distribution. This approach is currently being trialled at Fera Science through AHDB project FV 459 *Surveillance approaches, impact and epidemiology of virus diseases to improve management strategies*.

Over the longer term, HTS will inevitably be accepted as a stand-alone tool for detection and diagnosis in plant health laboratories. For this to happen, issues around technology validation and standardisation, and demonstrations of operator competence need to be overcome for regulatory authorities to develop trust in HTS approaches. A recent proficiency test focused on sequence analysis has demonstrated that this is also needed for bioinformatics analysis pipelines (Massart et al., 2019). The final challenge for the adoption of HTS will be to address assessing the plant health risk posed by the large volume of novel, previously undescribed viruses being detected. Rapidly sifting the high-risk pathogen candidates from the ubiquitous, but previously undescribed pathogens, or the persistent-lifestyle pathogens (Adams et al., 2018e). Predicting the host range and vectors of viruses based on sequence data motifs has been attempted for arboviruses (human and animal pathogens) (Babayan et al., 2018). Applying this approach to plant viruses will be more challenging given the broader range of hosts, and vectors which may be involved. For example, some sequence motifs are reported to be related to

biological characteristics, such as the DAG motif linked to aphid transmissibility in potyviruses (Harrison & Robinson, 1988). The presence of this motif has been used to predict aphid transmision of the ipomoviruses cassava brown streak virus, squash vein yellowing virus and coccinia mottle virus (Ateka et al., 2017). However, such predictive studies will need to be further underpinned by extensive characterisation of both the virus and vector to better understand the underlying drivers of the relative differences in vector efficiency. However, there are significant knowledge gaps which can only be addressed through experimental investigation, such as the example of the vectors of viruses of the genus *Torradovirus* discussed in section 1.2.5.3. where the aphid vectors could not be predicted based on prior knowledge.

1.3.1.2 Looking back to go forward

In working out the potential risks of novel viruses and gathering the essential data to underpin pest risk assessment there is also an untapped reserve of biological data which could be accessed, in terms of those viruses and virus isolates held in historic collections. In many cases these viruses were identified and biologically characterised in an era before access to either Sanger sequencing or HTS. Identifying viruses in collections which are linked to previous publications could be used to circumvent biological characterisation with access to these isolates' sequence data, such as those linked to the reports in *Ullucus tuberosus* (Fox et al., 2019). Work is ongoing to curate and sequence these historic isolates from collections (Adams et al., 2018a, Adams et al., 2018b, Adams et al., 2018c, Adams et al., 2018d, Fribourg et al., 2019). A coordinated international project starting in late 2019, Euphresco-"VirusCurate", will help to accelerate this effort, providing sequence data to support both taxonomy and risk assessment.

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2 PLANT VIRUSES AND VARROIDS IN THE UNITED KINGDOM: AN ANALYSIS OF FIRST DETECTIONS AND NOVEL DISCOVERIES FROM 1980 TO 2014

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3 USE OF NEXT-GENERATION SEQUENCING FOR THE IDENTIFICATION AND CHARACTERIZATION OF MAIZE CHLOROTIC MOTTLE VIRUS AND SUGARCANE MOSAIC VIRUS CAUSING MAIZE LETHAL NECROSIS IN KENYA

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Adams IP, Miano DW, Kinyua ZM, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richards R, Deb Nath P, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, and Boonham N. (2013) Use of next-generation sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology*, 62:4, pp741-749 [DOI: 10.1111/j.1365-3059.2012.02690.x]

4 CARROT YELLOW LEAF VIRUS IS ASSOCIATED WITH CARROT INTERNAL NECROSIS

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5 DIRECT TUBER TESTING FOR POTATO Y POTYVIRUS BY REAL-TIME RT-PCR AND ELISA: RELIABLE OPTIONS FOR POST-HARVEST TESTING?

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6 THE APPLICATION OF NEXT-GENERATION SEQUENCING FOR SCREENING SEEDS FOR VIRUSES AND VIROIDS

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Fox A, Adams IA, Hany U, Hodges T, Forde SMD, Jackson LE, Skelton A, Barton V (2015) The application of Next-Generation Sequencing for screening seeds for viruses and viroids, *Seed Science and Technology*, 43, 1-5. http://doi.org/10.15258/sst.2015.43.3.06

7 New aphid vectors and efficiency of transmission of Potato virus A and strains of Potato virus Y in the UK

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Fox A, Collins L, Macarthur R, Blackburn LF and Northing P. (2017) New aphid vectors and efficiency of transmission of Potato virus A and strains of Potato virus Y in the UK, *Plant Pathology*. 66, 2 pp325-335 doi: 10.1111/ppa.12561

8 APPENDIX 1. BIBLIOGRAPHY OF WORKS PUBLISHED

8.1 Peer reviewed publications

Vazquez-Iglesias I, Adams IP, Hodgetts J, Fowkes AR, Forde S, Ward R, Buxton-Kirk A, Kelly M, Santin-Azcona J, Skelton A, Harju V, Boonham N, Robinson R Clover G, Fox A, 2019. High throughput sequencing and RT-qPCR assay reveal the presence of rose cryptic virus-1 in the United Kingdom, *Journal of Plant Pathology*, 101:1171-1175

Fox A, Fowkes AR, Skelton A, Harju V, Buxton-Kirk A, Kelly M, Forde SMD, Pufal H, Conyers C, Ward R, Weekes R, Boonham N, Adams IP, 2019. Using High Throughput Sequencing in support of a plant health outbreak reveals novel viruses in *Ullucus tuberosus* (*Basellaceae*). *Plant Pathology,* 68, 576-587

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Fox A, Daly M, Nixon T, Brurberg MB, Blystad D, Harju V, Skelton A, Adams IP, 2013. First report of Tomato chlorotic dwarf viroid (TCDVd) in tomato in Norway and subsequent eradication. *New Disease Reports*, 27:8.

Adams I. P, Miano D. W, Kinyua Z. M, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richards R, Deb Nath P, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, and Boonham N, 2013. Use of next-generation sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology*, 62:4, 741-749

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8.2 Book Chapters

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Tomlinson J, Mumford R. published CABI, Wallingford, UK. ISBN 978 1 78064 147 8

8.3 Conference papers

Fox A, Monger W, Nixon T, Glover R, Barber P, 2009. Characterisation of an atypical isolate of Potato Virus M. *Aspects of Applied Biology: Potato viruses and their vectors*, 94, 35-40.

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8.5 Scientific opinion risk assessments

(External expert on behalf of the EFSA Panel on Plant Health)

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9 APPENDIX 2. CO-AUTHOR CONTRIBUTION STATEMENTS

9.1 Co-Author statement Prof. Rick Mumford, Chapter 2

Food Standards Agency
food.gov.uk

From the Head of Science, Evidence & Research
Prof. Rick Mumford FRSB

Tel: [redacted]
Email: [redacted]

Adrian Fox
Fera Science Ltd.
Sand Hutton
York
YO41 1LZ

Via Email: [redacted]

15th February 2019

Co-Author Statement

Title: Plant viruses and viroids in the United Kingdom: an analysis of first detections and novel discoveries from 1980-2014

Year: 2017

Authors: Adrian Fox, Rick Mumford

Journal: Virus Research

Personal Contribution:

- Co-formulation of the idea for the review (50% contribution to this aspect of the publication)
- Conducted literature review and data interpretation. Prepared figures (100% contribution)
- Drafted introduction, overview of records (results) and discussion. (80% contribution to this aspect of the publication)
    - Co-author drafted case study and provided review and partial re-drafting.
- First and corresponding author including response to reviewers.

Co-Author details:

Professor Rick Mumford	Head of Science, UK Food Standards Agency.	[redacted]

Foss House, Kings Pool,
1-2 Peasholme Green
York YO1 7PR

	Former Head of Science, Fera Science Ltd.	

Co-author statement: I can confirm that Adrian was the lead author on this paper with the contribution as described above. While we shared the concept development, it was Adrian that collated and analysed the data, produced the draft text and all the figures and tables.

9.2 Co-author coversheet Chapter 3

Title: Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya

Year: 2013

Authors: IP Adams, DW Miano, ZM Kinyua, A Wangai, E Kimani, N Phiri, R Reeder, V Harju, R Glover, U Hany, R Souza-Richards, P Deb Nath, T Nixon, **A Fox**, A Barnes, J Smith, A Skelton, R. Thwaites, R Mumford, N Boonham

Journal: Plant Pathology

Journal profile: The journal of the British Society of Plant Pathology. Publishes research papers and critical reviews on all aspects of plant pathology.

Personal Contribution:

- As the lead plant virologist, I designed the diagnostic strategy for sample analysis, Initiating the conventional diagnostic screening, including bioassay, ELISA and Electron microscopy, and identifying the samples as candidates for NGS screening. (60% of this aspect of work)
- Assessment of NGS data for presence of likely plant pathogens consistent with the observed symptoms, identification of the potential causal pathogens from these data (40% of this aspect of the work).
- Co-drafted relevant sections of the manuscript including methods and results for the conventional diagnostic screening. Reviewed the manuscript (approximately 15% of manuscript)

Co-Author details:

Dr IP Adams,	Senior molecular biologist, Fera Science Ltd	[redacted]
V Harju	Senior Virology Diagnsotician, Fera Science Ltd	[redacted]
R Glover	Former Bioinformatician, Fera Science Ltd	[redacted]

Dr R Souza-Richards	Specialist Advisor, Ministry for Primary Industries, New Zealand Former PhD Student, Fera Science Ltd	[redacted]
A Barnes	Senior Mycology Diagnostician, Fera Science Ltd	[redacted]
Dr J Smith	International Business Development Manager, Fera Science Ltd	[redacted]
A Skelton	Senior Virology Diagnostician, Fera Science Ltd	[redacted]

Please also see accompanying letters from:

Dr D. Miano, Dr R. Reeder, Ms. U Hany, Prof. P Deb-Nath, Mr. T Nixon, Prof. R. Mumford, Prof N. Boonham

The following co-authors did not respond to requests for co-author statements, despite repeated email contacts:

Mr Z. Kinyua, Dr A. Wangai, Mrs E Kimani, Mr N Phiri

The following co-author could not be contacted due to no forwarding details:

Dr R. Thwaites

9.3 Co-author statement from Dr D. W. Miano

UNIVERSITY OF NAIROBI
COLLEGE OF AGRICULTURE AND VETERINARY SCIENCES
FACULTY OF AGRICULTURE
DEPARTMENT OF PLANT SCIENCE AND CROP PROTECTION
P. O. Box 29053 00625 Kangemi, **NAIROBI,** TEL. 0202055129

14th May, 2019.

TO WHOM IT MAY CONCERN

Dear Sir/Madam,

Re: Contribution of Mr. Andrian Fox to the manuscript on identification of viruses causing MLN disease in Kenya

I write to confirm that I am a co-author with Mr. Andrian Fox, together with others, to the paper titled,

'Adams I. P, Miano D. W, Kinyua Z. M, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richards R, Deb Nath P, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, and Boonham N. (2013) Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing Maize Lethal Necrosis in Kenya. *Plant Pathology*, 62:4, pp741-749 [DOI: 10.1111/j.1365-3059.2012.02690.x]'

Adrian played a key role in designing the diagnostic strategy for sample analysis, initiating the conventional diagnostic screening using different techniques, and in identifying the samples as candidates for NGS screening. He also contributed significantly in the assessment of NGS data for presence of likely plant pathogens and identification of the potential causal pathogens. He co-drafted and reviewed relevant sections of the manuscript including methods and results for the conventional diagnostic screening.

The work reported in this paper was critical in identification and management of the causal agents of Maize lethal necrosis disease affecting maize in Kenya and in Eastern Africa, a new disease in the region that was causing high yield losses.

Yours sincerely,

[redacted]

Douglas W. Miano (Ph.D)
Senior Lecturer, Plant Virology and Biotechnology,
Department of Plant Science and Crop Protection,
University of Nairobi.
Tel. [redacted]
Email: [redacted]

9.4 Co-author statement from Dr R. Reader

Title: Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya

Year: 2013

Authors: IP Adams, DW Miano, ZM Kinyua, A Wangai, E Kimani, N Phiri, R Reeder, V Harju, R Glover, U Hany, R Souza-Richards, P Deb Nath, T Nixon, **A Fox**, A Barnes, J Smith, A Skelton, R. Thwaites, R Mumford, N Boonham

This letter is in support of the work carried out by Adrian Fox for the above paper. I can confirm that as the senior plant virologist on this investigation, Adrian took the lead in investigating the maize samples sent from Kenya. This was an complex and important investigaton as the diease was new to Kenya and was causing significant yield losses on a major export and subsustance crop (*pers.obs.*). The field symptoms strongly indicated virus involvement. However, the rapid development of the disease and the subsequent death of the plants was unusual and suggested perhaps a combination of viruses. Investigation of the literature suggested that this might be the viral complex known as maize lethal necrosis and investigations were conducted withthis hypothesis in mind, but also lookkng for other potential pathogens.

Adrian came up with a diagnostic testing strategy orientatied towards looking for putative agents that included bioassay, ELISA, Electron microscopy and Next Geneartaion sequencing. The NGS sequencer proved to be an effective tool for anysing the samples as both ELISA and sap inoculation tests proved inconclusive. Because the NGS produces many thousands of sequences, Adrian had to analysis the outputs looking for sequences of potetial candidate pathogens.

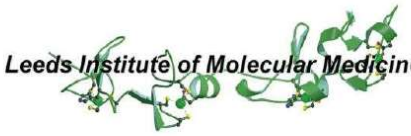
The final part of the study was the writing of the manuscript. I can confirm that Adrian undertook the writing of the methods and results sections and was involved in reviewing of he manuscript.

Yours Sincerely,

[redacted]

Robert Reeder

9.5 Co-author statement from Ms U Hany



UNIVERSITY OF LEEDS

08/05/2019

To Whom It may Concern

This is to certify that I was a co-author with Adrian Fox in the following papers when I used to work in Fera. I do agree with the following contributions from Adrian Fox in the study as well as in drafting the manuscripts.

1. Fox A, Adams I.A, Hany U, Hodges T, Forde S.M.D, Jackson L.E, Skelton A, Barton V (2015) The application of Next-Generation Sequencing for screening seeds for viruses and viroids. *Seed Science and Technology* 43 (3): 531-535(5)

   Personal Contribution:
   - Overall 40%
   - Experimental design including identification of sample seed lots (90% contribution to this aspect of work)
   - Post sequencing data analysis, including virus identification (30% contribution to this aspect of work)
   - Drafted manuscript, formulated figures (80% contribution to this aspect of work).
   - Lead and corresponding author including responding to reviewer's comments.

2. Adams IP, Miano DW, Kinyua ZM, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richards R, Nath PD, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, Boonham N (2013). Use of next-generation sequencing for the identification and characterization of Maize chlorotic mole virus and Sugarcane mosaic virus causing maize lethal necrosis in Kenya. *Plant Pathology* 62,741–749

   Personal Contribution
   - As the lead plant virologist, I designed the diagnostic strategy for sample analysis, initiating the conventional diagnostic screening, including bioassay, ELISA and Electron microscopy, and identifying the samples as candidates for NGS screening. (60% of this aspect of work)
   - Assessment of NGS data for presence of likely plant pathogens consistent with the observed symptoms, identification of the potential causal pathogens from these data (40% of this aspect of the work).
   - Co-drafted relevant sections of the manuscript including methods and results for the conventional diagnostic screening. Reviewed the manuscript (approximately 15% of manuscript)

I wish Adrian success in his PhD endeavour.

Yours faithfully,

[redacted]

Ms Ummey Hany

Next Generation Sequencing Facility
6.37, Clinical Sciences Building
St James's Campus Infrastructure and Facilities (SCIF)
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Leeds LS9 7TF
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F +44 (0) 113 343 8702

9.6 Co-author statement Prof. P Deb-Nath

DEPARTMENT OF PLANT PATHOLOGY: FACULTY OF AGRICULTURE
ASSAM AGRICULTURAL UNIVERSITY: JORHAT 785013

Dr Palash Deb Nath

Professor

Email: [redacted]

Mobile: [redacted]

Fax: [redacted]

To

Adrian Fox
CBiol SPHP MRSB
Principal Plant Virologist
Fera Science Ltd.

Dear Adrian Fox

I am happy to inform you that, I was co-author of two research papers as listed below, where in you made significant contributions in the line that are mentioned in the list. I agree with the claim that you made for these papers.

Paper Title	Year	Authors	Journal	Journal profile	Contributions made by Adrian Fox
Carrot yellow leaf virus Is Associated with Carrot Internal Necrosis	2014	Ian P Adams, Anna Skelton, Roy Macarthur, Tobias Hodges, Howard Hinds, Laura Flint, Palash Deb Nath, Neil Boonham, Adrian Fox	PloS one	The world's first multidisciplinary Open Access journal. The journal's publication criteria are based on high ethical standards and the rigor of the methodology and conclusions reported	- Hypothesis formulation. (100% of this aspect of study) - Initiated the study and secured the funding. (100% of this aspect of the study) - With statistical support, Adrian designed the sampling and sub-sampling strategy used in the study. (60% of this aspect of the study) - Led on the data analysis regarding biological plausibility of the findings and analysing the relative virus incidences. (90% this aspect of the study) - Drafted introduction, methods, results and discussion. Prepared figures. (80% of this aspect

					of the study) - Final and corresponding author responsible for submission, responding to reviewer's comments and suggestions.
Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya	2013	IP Adams, DW Miano, ZM Kinyua, A Wangai, E Kimani, N Phiri, R Reeder, V Harju, R Glover, U Hany, R Souza-Richards, P Deb Nath, T Nixon, **A Fox**, A Barnes, J Smith, A Skelton, R. Thwaites, R Mumford, N Boonham	Plant Pathology	The journal of the British Society of Plant Pathology. Publishes research papers and critical reviews on all aspects of plant pathology	- As the lead plant virologist, Adrian designed the diagnostic strategy for sample analysis, Initiating the conventional diagnostic screening, including bioassay, ELISA and Electron microscopy, and identifying the samples as candidates for NGS screening. (60% of this aspect of work) - Assessment of NGS data for presence of likely plant pathogens consistent with the observed symptoms, identification of the potential causal pathogens from these data (40% of this aspect of the work). - Co-drafted relevant sections of the manuscript including methods and results for the conventional diagnostic screening. Reviewed the manuscript (approximately 15% of manuscript)

(Palash Deb Nath)

9.7 Co-author statement Mr. T Nixon

Animal & Plant Health Agency

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www.gov.uk/apha

Adrian Fox
Principal Plant Virologist, Fera Science Ltd
Sand Hutton
York
YO41 1LZ

8th May 2019

Dear Adrian Fox,

Re: Coauthor Supporting Statement

Thank you for your email on the 30th April 2019 regarding a co-author statement.

I can confirm that we were co-authors on the following paper: IP Adams, DW Miano, ZM Kinyua, A Wangai, E Kimani, N Phiri, R Reeder, V Harju, R Glover, U Hany, R Souza-Richards, P Deb Nath, T Nixon, A Fox, A Barnes, J Smith, A Skelton, R. Thwaites, R Mumford, N Boonham, 2013, Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya, Plant Pathology.

As you were the lead virologist I can confirm your personal contribution was:

- To design the diagnostic strategy for sample analysis, initiating the conventional diagnostic screening, including bioassay, ELISA and Electron microscopy, and identifying the samples as candidates for NGS screening. (60% of this aspect of work)
- Complete the assessment of NGS data for presence of likely plant pathogens consistent with the observed symptoms, identification of the potential causal pathogens from these data (40% of this aspect of the work)
- Co-draft relevant sections of the manuscript including methods and results for the conventional diagnostic screening. Reviewed the manuscript (approximately 15% of manuscript)

Yours sincerely,

[redacted]

Tom Nixon, Senior Plant Health & Seed Inspector, Tel No: [redacted] Email: [redacted]

9.8 Co-author statement Prof. R Mumford, Chapter 3

Food Standards Agency
food.gov.uk

From the Head of Science, Evidence & Research
Prof. Rick Mumford FRSB

Tel: [redacted]
Email: [redacted]

Adrian Fox
Fera Science Ltd.
Sand Hutton
York
YO41 1LZ

Via Email: [redacted]

15th February 2019

Co-Author Statement

Title: Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya

Year: 2013

Authors: IP Adams, DW Miano, ZM Kinyua, A Wangai, E Kimani, N Phiri, R Reeder, V Harju, R Glover, U Hany, R Souza-Richards, P Deb Nath, T Nixon, **A Fox**, A Barnes, J Smith, A Skelton, R. Thwaites, R Mumford, N Boonham

Journal: Plant Pathology

Journal profile: The journal of the British Society of Plant Pathology. Publishes research papers and critical reviews on all aspects of plant pathology.

Personal Contribution:

- As the lead plant virologist, I designed the diagnostic strategy for sample analysis, Initiating the conventional diagnostic screening, including bioassay, ELISA and Electron microscopy, and identifying the samples as candidates for NGS screening. (60% of this aspect of work)
- Assessment of NGS data for presence of likely plant pathogens consistent with the observed symptoms, identification of the potential causal pathogens from these data (50% of this aspect of the work).
- Co-drafted relevant sections of the manuscript including methods and results for the conventional diagnostic screening. Reviewed the manuscript (approximately 15% of manuscript)

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Professor R Mumford	Head of Science, UK Food standards Agency Former Head of Science, Fera Science Ltd.	[redacted]

Co-author statement: I can confirm that Adrian's contribution on this this paper was as described above. As Fera's lead virology diagnostician, he had a critical and central role in the design, interpretation, delivery and interpretation of the core data used in the production of the paper. He was also active in the manuscript production. Indeed, in hindsight, I would question his position in the authorship list.

9.9 Co-Author statement Prof. Neil Boonham

Newcastle University

School of Natural & Environmental Sciences

Head of School
Professor Rob Edwards

Newcastle University
Agriculture Building
Newcastle upon Tyne
NE1 7RU United Kingdom

18th February 2019

RE: Manuscript contributions

I can confirm as a co-author on the manuscripts detailed below, that Adrian Fox made significant inputs into each study as detailed in the attached paper contribution documents. For the study on carrots Adrian was the overall lead for the work, from concept, through funding to the research itself and publication. For the maize study Adrian was a key member of the team and made invaluable contributions, both technically on the work as well as in drafting and revision of the manuscript. Both papers have made significant contributions to the field firstly, in terms of technology/diagnostic development and the acceptance of these methods in testing labs around the world. Furthermore, each study made very significant specific impact in terms of the diseases under study. Carrot root necrosis is a disease that has caused problems for the carrot industry in the UK for 15-20 years, without knowledge of the causal agent, there was little prospect for the industry to identify solutions. The outbreak of maize lethal necrosis in East Africa in 2012 was a rapidly spreading and uncharacterised syndrome in a staple crop relied upon by millions of smallholder farmers, the rapid resolution of the causal agent led to the development of potential solutions for the region.

Adams, I.P., Skelton, A., Macarthur, R., Hodges, T., Hinds, H., Flint, L., Nath, P.D., Boonham, N., & Fox, A.J. (2014). *Carrot yellow leaf virus* Is Associated with Carrot Internal Necrosis. PLoS ONE 9(11): e109125. https://doi.org/10.1371/journal.pone.0109125

Adams IP, Miano DW, Kinyua ZM, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richards R, Deb Nath P, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, Boonham N, 2013. Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya. *Plant Pathology* **62**, 741-749. [http://dx.doi.org/10.1111/j.1365-3059.2012.02690.x]

Yours faithfully

[redacted]

Professor Neil Boonham

School of Natural and Environmental Sciences

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9.10 Co-author coversheet Chapter 4

Paper Title: Carrot yellow leaf virus Is Associated with Carrot Internal Necrosis

Year: 2014

Authors: Ian P Adams, Anna Skelton, Roy Macarthur, Tobias Hodges, Howard Hinds, Laura Flint, Palash Deb Nath, Neil Boonham, Adrian Fox

Journal: PloS one

Journal profile: The world's first multidisciplinary Open Access journal. The journal's publication criteria are based on high ethical standards and the rigor of the methodology and conclusions reported.

Personal Contribution:

- Hypothesis formulation. (100% of this aspect of study)
- Initiated the study and secured the funding. (100% of this aspect of the study)
- With statistical support, I designed the sampling and sub-sampling strategy used in the study. (60% of this aspect of the study)
- Led on the data analysis regarding biological plausibility of the findings and analysing the relative virus incidences. (90% this aspect of the study)
- Drafted introduction, methods, results and discussion. Prepared figures. (80% of this aspect of the study)
- Final and corresponding author responsible for submission, responding to reviewer's comments and suggestions.
-

Co-author details:

Ian P Adams	Senior molecular biologist, Fera Science Ltd	[redacted]
Anna Skelton	Senior Virology Diagnostician, Fera Science Ltd	[redacted]
Roy Macarthur	Senior Statistician, Fera Science Ltd	[redacted]

Please also see accompanying co-author statements from:

Dr T. Hodges, Mr H. Hinds, Prof. P. Deb-Nath and Prof. N. Boonham

Despite repeated attempted email contacts the following co-author did not respond to requests for a co-author statement: Dr L. Flint

9.11 Co-author statement Dr T. Hodges

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29th April 2019

Confirmation of Author Contributions – Adrian Fox

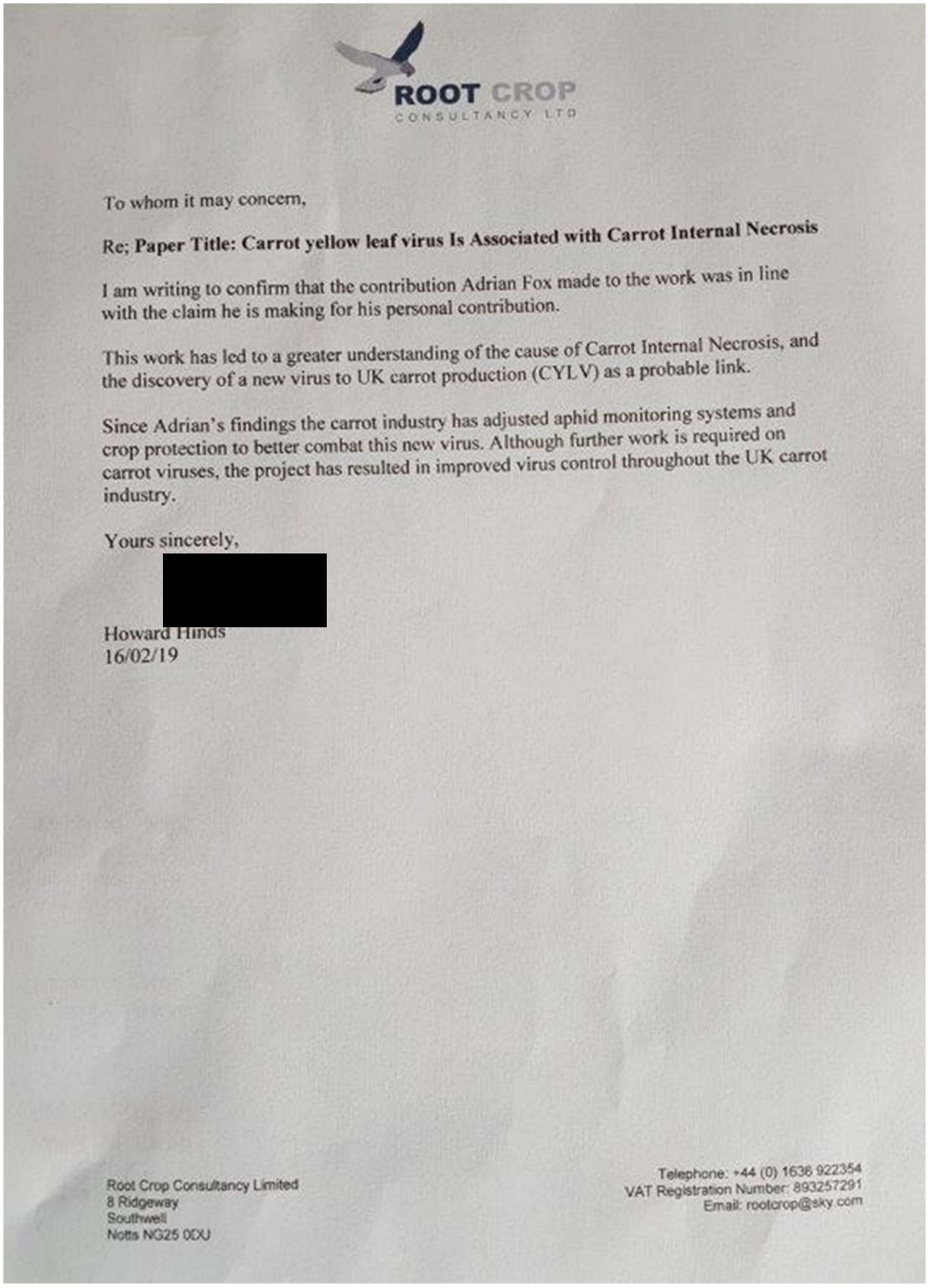
To Whom It May Concern: I hereby confirm that the attached Contribution Statements accurately describe the contributions made by Adrian Fox to two papers on which I am Co-Author, "Carrot yellow leaf virus Is Associated with Carrot Internal Necrosis" (*PLOS ONE*, 2014) and "The application of Next-Generation Sequencing for screening seeds for viruses and viroids" (*Seed Science and Technology*, 2015).

Yours truly,

[redacted]

Dr Toby Hodges

9.12 Co-author statement from Mr H. Hinds



ROOT CROP
CONSULTANCY LTD

To whom it may concern,

Re; **Paper Title: Carrot yellow leaf virus Is Associated with Carrot Internal Necrosis**

I am writing to confirm that the contribution Adrian Fox made to the work was in line with the claim he is making for his personal contribution.

This work has led to a greater understanding of the cause of Carrot Internal Necrosis, and the discovery of a new virus to UK carrot production (CYLV) as a probable link.

Since Adrian's findings the carrot industry has adjusted aphid monitoring systems and crop protection to better combat this new virus. Although further work is required on carrot viruses, the project has resulted in improved virus control throughout the UK carrot industry.

Yours sincerely,

[redacted]

Howard Hinds
16/02/19

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9.13 Co-author coversheet Chapter 5

Title: Direct tuber testing for Potato Y potyvirus by real-time RT-PCR and ELISA: reliable options for post-harvest testing?

Year: 2005

Authors: Adrian Fox, Fiona Evans, Isla Browning

Journal: EPPO Bulletin

Personal Contribution:

- Experimental design, including methodology for comparative testing and time-course study. (50% contribution to this aspect of the study)
- Practical work including plant raising (growing on of potato eye plugs and whole tubers),(100% contribution to this aspect of the study)
- ELISA and molecular diagnostics.(100% of the ELISA testing and 50% of the molecular testing within the study)
- Result analysis of both ELISA and molecular testing.(75% contribution to this aspect of the study)
- Manuscript preparation including drafting, formatting and preparation of figures.(90% contribution to this aspectof the study)
- As first and corresponding author I was also responsible for the responses to reviewers.

Co-Author Details, signed as supporting evidence of authorship contribution detailed above:

Dr Fiona Evans	Former molecular diagnostician, SASA, Edinburgh, UK	[redacted]
Isla Browning	Head of Virology, (Retired) SASA, Edinburgh	[redacted]

9.14 Co-author coversheet Chapter 6

Title: The application of Next-Generation Sequencing for screening seeds for viruses and viroids

Year: 2015

Authors: Adrian Fox, Ian Adams, Ummy Hany, Tobias Hodges, Stephen Forde, Lucy Jackson, Anna Skelton, Victoria Barton

Journal: Seed Science and Technology

Personal Contribution:

- Overall c. 40%
- Experimental design including identification of sample seed lots (90% contribution to this aspect of work)
- Post sequencing data analysis, including virus identification (30% contribution to this aspect of work)
- Drafted manuscript, formulated figures (80% contribution to this aspect of work).
- Lead and corresponding author including responding to reviewer's comments.

Co-author details:

Ian Adams	Senior molecular biologist, Fera Science Ltd	[redacted]
Stephen Forde	Virus Diagnostician, Fera Science Ltd	[redacted]
Lucy Jackson	Former Virus Diagnostician Fera Science Ltd	[redacted]
Anna Skelton	Senior Virus Diagnostician, Fera Science Ltd	[redacted]
Victoria Barton	Seeds and Exports Team Leader, Fera Science Ltd	[redacted]

Please also see accompanying co-author statements from:

9.15 Co-author coversheet Chapter 7

Title: New aphid vectors and efficiency of transmission of *Potato virus A* and strains of *Potato virus Y* in the UK

Year: 2017

Authors: Adrian Fox, Larissa Collins, Roy Macarthur, Lisa Blackburn, Phil Northing

Journal: Plant Pathology

Journal profile: The journal of the British Society of Plant Pathology. Publishes research papers and critical reviews on all aspects of plant pathology.

Journal IF (5yr): 2.525

H index (SJR): 68

Citations: 2 (ResearchGate) 4 (Google Scholar)

Personal Contribution:

- Experimental design: Adapted the method from Verbeek et al (2010) for assessment of PVA and included PVY strains and a common reference aphid biotype from both studies to validate results.
- Oversaw ELISA testing of virus source material and first repetitions of testing to validate visual virus assessments. Carried out visual virus assessments of receiver plants.
- Calculated REFs for comparison with previous reports. Consulted with statistician for further data analysis.
- Conducted literature search.
- Drafted all aspects of the paper.
- As first and corresponding author I also dealt with the editorial process, such as responding to reviewer's comments.

Dr Larissa Collins	Entomolgist Fera Science Ltd	[redacted]
Roy Macarthur	Statistician Fera Science Ltd	[redacted]
Lisa Blackburn	Entomologist Fera Science Ltd	[redacted]
Phil Northing	Head of Plant Programme Fera science Ltd	[redacted]